PATENT APPLICATION

Our Docket No. 20040351.DIB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Re App	:	Carol W. Readhead et al. :	November 30, 2006
S.N.	:	10/008,385 :	Art Unit 1632
Filed	:	November 12, 2001 :	Examiner Joanne Hama
For	:	TRANSFECTION, STORAGE AND TRANSFER OF MALE GERM CELLS FOR GENERATION OF TRANSGENIC SPECIES & GENETIC THERAPIES	

LETTER

Mail Stop APPEAL BRIEF - PATENTS
Commissioner For Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This paper is submitted in response to an official communication in the form of a Notification of Non-Compliant Appeal Brief Under 37 CFR 41.37 dated November 27, 2006. That Notification has a shortened-statutory period for response of one month or thirty days so that a reply is due by December 27, 2006.

The Brief was cited for failure to include information as to where in the record evidence was entered by the Examiner that was included in an Evidence Appendix. Also, one reference (Kim et al, 1997) was missing from two of the three Brief copies.

Accordingly, enclosed is a Second Amended Brief in which references have been added both to the body of the Brief and to the Evidence Appendix to refer to the record with respect to the

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SECOND AMENDED APPEAL BRIEF FOR THE APPELLANTS

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I. REAL PARTY IN INTEREST

The real parties in interest are Imperial College Innovations Ltd., Exhibition Road, Sherfield Building, London, United Kingdom SW7 2AZ, by virtue of mesne Assignments, including Assignments from certain inventors and other earlier assignees and Cedar-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, California 90048, by assignment from inventor, Carol W. Readhead. The present application is a division of Serial No. 09/191,920 filed November 13, 1998, now Patent No. 6,316,692 which, in turn, claimed the benefit of Provisional Application 60/065,825 filed November 14, 1997. By

virtue of being a division of what is now Patent No. 6,316,692, the Assignment of that application inures to the present application. That patent has some seventeen total assignments of record and a printout of the Patent Assignment Abstract of Title for that patent is attached hereto as Appendix A.

II. RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences known to the owner of the subject application, the owner's legal representatives, or the inventors which will directly affect or be directly affected by or should have a bearing on the Board of Patent Appeals and Interferences in the pending appeal to the present knowledge and belief of the undersigned.

III. STATUS OF THE CLAIMS

The present application was filed on November 12, 2001 as Serial No. 10/008,385 and has undergone several Office Actions and amendments. The original claims 1-134, originally filed in parent application 09/191,920, were canceled in a Preliminary Amendment in which claims 135-155 were added. Since then, claims 156-160 have been added and claims 136, 139 and 144 have been canceled. The Office Action from which this Appeal was taken was issued on July 26, 2005, an earlier Final Action dated April 11, 2005, having been vacated by the Examiner. An After-Final Amendment was submitted dated September 16, 2005 and an

Advisory Action was issued on October 13, 2005. The Advisory Action indicated that for purposes of appeal, the After-Final Amendment would be entered and contained a further explanation of how the newer amended claims would be rejected. On January 26, 2006, a Notice of Appeal was lodged for the present appeal in which claims 135, 137-138, 140-143 and 145-160 stand rejected and no claim has been allowed.

Thus, the present status of all the claims is as follows:

1-134. (Canceled)

135. (Rejected)

136. (Canceled)

137-138. (Rejected)

139. (Canceled)

140-143. (Rejected)

144. (Canceled)

145-160. (Rejected)

What Appellants believe to be a true copy of the claims presently under appeal appears in Appendix B attached to this Brief.

IV. STATUS OF AMENDMENTS

All amendments submitted in this application are believed to have been entered and are presently considered to be of record.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The field of transgenics was initially developed to understand the action of a single gene in the context of the whole animal and phenomena of gene activities, expression and interaction. This technology has been used to produce models for various diseases in humans and other animals. Transgenic technology is amongst the most powerful tools available for the study of genetics and the understanding of genetic mechanisms and function. It is also being used to study the relationship between genes and diseases. Many diseases are caused by a single genetic defect or are the result of complex interaction genes and environmental agents. The understanding of such interactions is of prime importance for the development of therapies, such as gene therapy and drug therapies, and also treatments such as organ transplantation.

Transgenesis has played a part in improving breeds of non-human mammals such as livestock. Historically, transgenic animals have been produced almost exclusively by microinjection of the fertilized egg. The microinjected fertilized eggs are then transferred to the genital tract of a pseudopregnant female. The generation of transgenic animals by this technique is generally reproducible and, for this reason, little has been done to improve on it. That technique, however, requires large

numbers of fertilized eggs and so has proved to be quite inefficient in producing transgenic animals.

The present claimed invention relates to the field of transgenics and gene therapy and, more specifically, to an *in vitro* method for incorporating at least one polynucleotide including a desired trait into a male germ cell. By means of the claimed invention, male germ cells are transfected with at least one polynucleotide encoding a gene product in operable linkage with a promoter integrated in a viral vector which enables the polynucleotide to incorporate into the germ cell. Germ cells in which the polynucleotide is incorporated into the genome of the germ cell can then be isolated for further use such as being returned to the testes under suitable conditions where they will be spontaneously repopulated.

The application contains a lone independent claim, method claim 135, which enumerates four actions to a method for incorporating at least one polynucleotide encoding a desired trait into a male germ cell.

In part (a), a male germ cell selected from a group of non-human vertebrate species is obtained. Reference may be had to the specification, for example, at page 4, lines 6-9.

Thereafter, in the second action of the method (b), the selected male germ cell is transfected *in vitro* with at least

one polynucleotide encoding a gene product in operable linkage with a promoter which is included in a virus or virus-derived DNA. This is done in the presence of a gene delivery mixture including at least one transfecting agent and, optionally, a polynucleotide encoding a genetic selection marker. Reference is made to page 5, lines 5-15 and lines 22-25; also at page 10, lines 6; page 11, line 22; example 10, page 22, line 12; page 23, line 14.

This is followed by (c), allowing the polynucleotide encoding a gene product to be taken up by and released into the germ cell. This is described by material found throughout the specification, including page 5, lines 5-15.

Finally, in (d), the cells in which the polynucleotide has been successfully incorporated into the genome are selected for further use. Reference is made to page 4, lines 10-12; also in example 10 at page 23, lines 2-14, for example.

It is believed that the material in the dependent claims is also well-described throughout the specification, for example a listing of viruses can be found at page 10, lines 28-page 11, line 11 for claim 140. A listing of a group of non-human mammals which can be used as in claim 151 is found at page 12, lines 12-20. For claim 160, see, for example, page 14, line 21 and page 15, line 14.

It is believed that the claimed subject matter is adequately described for one to clearly interpret the claims and references any needed further descriptive material.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The statutory provision of 35 U.S.C. § 103(a) forms the sole legal basis for the rejection of claims 135, 137-138, 140-143 and 145-160. Two literature references are relied upon by the Examiner with respect to claims 135, 137-138, 140-143 and 145-159. They include:

Brinster and Zimmermann (PNAS, USA, 91:11298-11302, 1994)

Vogel and Sarver (Clinical Microbiology Reviews, 8:406-410, 1995)

With respect to claim 160, in addition to the above-cited references, the Examiner has added an additional reference in the form of a paper by Wivel and Walters (Science, 262:533-538, 1993).

In view of the foregoing, generally, the only issue to be decided on appeal is whether either of the combinations of references cited by the Examiner demonstrate that the subject matter of the present claims fails to meet the requirement for patentability imposed by 35 U.S.C. § 103.

In other words, should the rejection of claims 135, 137-138, 140-143 and 145-159 under 35 U.S.C. § 103(a) as being

unpatentable over Brinster and Zimmermann (1994) in view of Vogel and Sarver (1995); and the rejection of claim 160 under 35 U.S.C. § 103(a) as being unpatentable over Brinster and Zimmermann (1994) and Vogel and Sarver (1995) in view of Wivel and Walters (1993) stand?

VII. ARGUMENTS

A. Grouping of Claims

Appellants believe that each and every claim should stand or fall on its own merits and that the limitations of each should be considered separately. The rejections based on 35 U.S.C. § 103 have been applied to all of the claims. However, the dependent claims, when contrasted with independent claim 135, incorporate additional features which provide further support for their patentability. Accordingly, it is the belief of the Appellants that each and every claim should have the ability to stand or fall on its own merits and that the limitations of each should be considered separately. While the main thrust of the arguments will be directed to the independent claim at issue, the dependent claims add limitations and define combinations that should be considered on their own merits.

For example, many of the dependent claims require certain germ cells, viral vectors, retro viral vectors, transfecting agents, genetic selection markers, etc.

B. The Cited Art

(1) Brinster and Zimmermann (1994), (Brinster et al)

Brinster et al report that stem cells isolated from testis of donor male mice will repopulate sterile testis when injected into seminiferous tubules. They further merely suggest at page 11298, column 2:

> "Because of the unique characteristics and potential of stem cell spermatogonia, the ability to recover these cells, manipulate them *in vitro*, and transfer them to another testis would provide a valuable technique to study the process of spermatogenesis. Furthermore, modifications of these cells prior to transfer could influence the development of eggs fertilized by spermatozoa arising from the altered stem cells. We describe here the requisite first step in this approach, a method to transplant testis cells from one male to another and we demonstrate that spermatogenesis occurs from donor cells in the recipient male." (emphasis added)

Another pertinent reference occurs at page 11301, second column, last paragraph:

> "If spermatogonia can be cultured and manipulated -- e.g., via targeted homologous recombination of DNA

sequences -- and individual modified clones of cells can be selected in a manner similar to embryonic stem cells (18, 19), then these cells could be used to create mice with germline modifications." (emphases added).

The reference does not disclose or teach actual modification of the isolated stem cells and merely suggests the possible advantages of stem cell *in vitro* manipulation, as in the quote first above. With reference to the second quote, the suggestions seem somewhat speculative and there is clearly nothing contained in the reference that would suggest to one skilled in the art how this might be accomplished.

(2) Vogel and Sarver (1995), (Vogel et al)

The Vogel et al reference is directed to nucleic acid vaccines. The reference does teach that viral vectors, including retroviral vectors, provide an avenue for introducing foreign DNA into a cell using direct *in vivo* injection of nonreplicating retroviral particles and that "genes transferred via retroviral vectors are inserted into the host chromosome, thereby insuring the perpetuity of the genetic information in the target cells" (page 408, second column, first paragraph).

That reference, however, does not deal in any manner with *in vitro* manipulation of male germ cells. Furthermore, there is no teaching that there is a requirement for the nucleic acid to be in a viral vector or for it to integrate into the chromosome in order to produce an immune response.

(3) Wivel and Walters (1993), (Wivel et al)

This reference deals with gene modification and disease prevention and discusses medical and ethical perspectives. Wivel et al teach that there are some human genetic diseases which are candidates for genetic intervention in the manner of correcting or preventing genetic deficiencies. They describe human diseases that would be candidates for prevention by germ-line gene modification. These include Lesch-Nyhan syndrome, Tay-Sachs disease and Metachromatic Leukodystrophy.

C. Authorities and Arguments

The rejections under appeal here are all based on a plurality of references. In determining the propriety of a rejection under 35 U.S.C. § 103 based on a plurality of references, it is well settled that the obviousness of an invention cannot be established by combining the teachings of the several pieces prior art absent some teaching, suggestion or incentive in the art itself supporting the combination. See *In re Fine*, 837 F.2d 1071, 5 U.S.P.Q. 2d 1596 (Fed. Cir. 1988). A test for

obviousness is what the combined teachings of the references, taken as a whole, would have suggested to those having ordinary skill in the art. See *In re Kaslow*, 707 F.2d 1366, 217 U.S.P.Q. 1089 (Fed. Cir. 1983).

During the patent examination process, the U.S. Patent Office bears the initial burden of presenting a *prima facie* case of unpatentability. See *In re Oetiker*, 977 F.2d 1443, 24 U.S.P.Q. 2d 1443 (Fed. Cir. 1992). When the U.S. Patent Office fails to meet this burden, the appellant is entitled to the patent. However, when a *prima facie* case is made, the burden then shifts to the applicant to come forward with evidence and/or arguments supporting patentability to rebut the *prima facie* case. Patentability *vel non* is then determined on the entirety of the record, by a preponderance of the evidence and the weight of the argument. See *In re Paisecki*, 745 F.2d 1468, 223 U.S.P.Q. 785 (Fed. Cir. 1984).

The initial burden of establishing a *prima facie* case of obviousness thus rests upon the Examiner and that burden can only be satisfied by showing that objective teachings in the prior art or knowledge generally attributed to one of ordinary skill in the art would have led such an individual to combine the relevant teachings of the cited references. It is also well settled that it is error to reconstruct the appellants' claimed

invention from the prior art by using the appellants' claim as a "blueprint". When prior art references require selective combination to render a subsequent invention obvious, there must be some definitive reason for the combination to be made other than the hindsight obtained from the invention itself. See *Interconnect Planning Corp. v. Feil*, 774 F.2d 1132, 227 U.S.P.Q. 543 (Fed. Cir. 1985). "One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to depreciate the claimed invention." In re *Fine*, supra (Fed. Cir. 1988).

"To prevent the use of hindsight based on the invention to defeat patentability of the invention, this court requires the examiner to show a motivation to combine the references that create the case of obviousness". In re *Rouffet*, 149 F.3d 1350, 47 U.S.P.Q. 2d 1453 (Fed. Cir. 1998). "[T]he suggestion to combine requirement stands as a critical safeguard against hindsight analysis and rote application of the legal test for obviousness." In re *Rouffet*, supra.

In analyzing whether claimed subject is properly rejected under 35 U.S.C. § 103 based upon a combination of prior art references, two factors must be considered: (1) whether the prior art would have suggested to one of ordinary skill in the art that they should make the claimed composition or device, or

carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success. In re *Vaeck* 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed. Cir. 1991).

It has further long been well known and important to note that obviousness to try a particular approach or to undertake particular research does not make the results obvious. See, for example, in re Tomlinson et al 150 USPQ (CCPA 1966, 623).

For the reasons discussed and those given below, appellants will show that the prior art references cited by the Examiner do not suggest the invention as a whole claimed in the subject application.

Even assuming that the asserted combinations are proper, the claims of appellants' application define an invention that is believed to be neither taught nor suggested by the references relied upon by the Examiner. The inventors have probed the strengths and weaknesses of the prior art and discovered an improvement in versatility and simplicity that has escaped those who came before. This is indicative of unobviousness rather than obviousness. *Fromsen v. Anitec Printing Plates, Inc.*, 132 F.3d 1437, 45 U.S.P.Q. 2d. 1269 (Fed. Cir. 1997).

Brinster et al (1994) in view of Vogel et al (1995)

Based on the final rejection of July 26, 2005 and the Advisory Action dated October 13, 2005, it is the Examiner's position that the teaching of Brinster et al provides motivation for an artisan to use spermatogonia to introduce germ-line changes. Furthermore, the Examiner states at the bottom of page 3 of the Advisory Action that the fact that Brinster et al discussed the issue suggests reasonable expectation of success that one could arrive at the claimed invention. To this, the Examiner asserts that the motivation to the artisan to use retroviral vectors is provided by Vogel et al. Appellants believe this position cannot properly be sustained for reasons of record and for reasons presented here.

The disclosure of Brinster & Zimmermann provides no indication of how to introduce genetic material into male germ cells whereas, in contrast, the present claims are restricted to the use of viral vectors which allow the polynucleotide to incorporate into the germ cell. Brinster et al provide technical teaching only with respect to the transfer of stem cells isolated from testes of donor mice to repopulate sterile testes of recipient mice. There is no technical teaching of the transfection of the male germ cells, and certainly no suggestion that this be done using integrating viral vectors. In contrast,

to the extent that Brinster et al discloses anything of relevance, it suggests targeted homologous recombination of DNA sequences as evidenced in the second passage quoted above.

Further, as has been noted with respect to even that passage, the use of the words "if", "then", and "could" suggests mere speculation on the part of the authors as to future endeavors that could be tried and also indicate that they have no insight as to how this would be accomplished. At the time of the article, it was also clear that the culture of male germ cells was difficult and that it was difficult to retain the cells' integrity and male germ cell character in cultures.

Secondly, even if those skilled persons were to consider attempting to manipulate spermatogonia, they are specifically taught to do so "*via targeted homologous recombination of DNA sequences*". As is clear from Capecchi (1989, Trends in Genetics, 5:70-76; (also cited by the Examiner in an Action dated April 6, 2005) - See Evidence Appendix C, this is totally different from the use of integrating viruses (see Figure 5 of Capecchi on page 75 which highlights the differences in these approaches). In fact, the whole thrust of Capecchi is to allow gene targeting in embryonic stem (ES) cells which requires homologous recombination between DNA sequences residing in the

chromosome and newly introduced DNA sequences (see Abstract on page 70 of Capecchi).

Thus, Brinster et al, to the extent that it may be relevant, suggests trying homologous recombination and not the use of viral vectors which integrate in the genome. Furthermore, Brinster et al discloses only *targeted homologous recombination:* integration of virus or virus-derived DNA is not targeted.

Thirdly, Brinster et al gives two specific references (References 18 and 19) for the genetic modification contemplated. Reference 18 is Capecchi (above) which is directed at gene targeting. Reference 19, Smithies (1993) Trends in Genetics 9:112-116, (submitted by applicants in a paper dated September 16, 2005) (abstract cited and included in Appendix C attached) is a review of animal models of human genetic disease and, as can be seen from its abstract, relates to gene targeting in ES cells and not the use of viruses in male germ cells.

Additional evidence of record exists in other references previously cited by the Examiner in an Action originally dated October 6, 2003 and since withdrawn including Kim et al (Mol. Reprod. Dev., 1997, 46:515-526) and Bachiller et al (Mol.

Reprod. Dev., 1991, 30:194-200). These references are also included in Appendix C.

The evidence of Kim et al is that using a standard transfection procedure, i.e., liposome/DNA complexes, spermatozoa cannot be transfected so that the DNA is incorporated into their chromosome DNA (see page 519, column 1, lines 4-7).

The evidence of Bachiller et al is that when using a standard transfection procedure, i.e., liposome/DNA complexes, to transfect sperm, the sperm does not generate transgenic animals (see last sentence of the Abstract).

Thus, the evidence of record is that there is no reasonable expectation of success in doing what the Examiner alleges is an obvious thing to do.

The present claims, on the other hand, are restricted to the polynucleotide to be transfected into the male germ cells being comprised in a virus or virus-derived DNA and germ cells being selected in which the polynucleotide has incorporated into the genome.

This primary reference has been combined with Vogel et al which is said to teach retrovirus-mediated gene transfer and to teach using a retrovirus as a way of introducing a retroviral vector as a means of stably introducing a gene into the host

chromosome. The Examiner further asserts that the integration using a retroviral vector insures perpetuity of the genetic information in the target cells.

In this regard, the appellants maintain their position that Vogel et al is in a technical field sufficiently different, i.e., nucleic acid vaccines, from the technical field of Brinster et al and the present invention that the skilled artisan in the field of interest would not be led to consult it.

Even if the combination of Brinster et al and Vogel et al were proper, it does not add information that is more relevant to the patentability of the present claims. The Vogel et al reference is directed to nucleic acid vaccines which has nothing to do with making transgenic animals. The reference is believed to contain a possible inference to try an approach, but only in a different field.

Moreover, with respect to fundamental differences between what is taught or suggested by Vogel et al and any motivation to use viral DNA or viral derived DNA, it is noted at the outset that nucleic acid vaccines work by the nucleic acid being able to express a polypeptide which acts as an antigen in an immune response. There is no requirement for the nucleic acid to be in a viral vector or for it to integrate into the chromosome in order to do this. Thus, it is but one possible approach.

This is plain from the section entitled "Nucleic Acid Vaccine Development" on page 406 in which it is made clear that (1) DNA expression vectors in cationic lipid vesicles can be used or (2) naked plasmid DNA vectors. The work of Davis et al (Reference 5 on page 407, column 1) demonstrates that, at least in some tissues (e.g. regenerating muscle), recombinant plasmid and adenoviral vectors (non-integrating) are superior to retroviral vectors (integrating).

Given the above, one can only conclude that there is nothing whatsoever in Vogel et al which gives any guidance as to which, if any, of these systems would be applicable to the genetic manipulation of male germ cells. This is not surprising since Vogel et al is in such an unrelated field.

Furthermore, it appears that the present rejections represent an analysis based on an impermissible hindsight reconstruction of the invention by hindsight. The Examiner has attempted to reconstruct the claimed invention not from a position of what the skilled person would do in modifying the teachings of Brinster et al (which, if anything, is to attempt to use homologous recombination on spermatogonial cells), but impermissibly using knowledge gleened from the claimed invention and casting around to try to find a paper which "fills in the gap" of Brinster et al. Vogel et al cannot do this since, as

discussed, it is in a different field (vaccines) and so would not be considered by the skilled person in any event. Furthermore, Vogel et al, in any event, discloses a range of ways in which one could potentially genetically manipulate cells without suggesting any particular one.

It should be remembered that the authors of Kim et al (which has been discussed previously), who presumably were aware of a desire that any genetic manipulation of the cells was carried to the next generation, used a liposome-mediated gene delivery approach. It is noteworthy that Kim et al was published in 1997, i.e., immediately before the claimed priority date and some 2-3 years later than Brinster et al, thus indicating again that the use of integrating viral vectors was not obvious to the skilled person.

Brinster et al (1994) and Vogel et al (1995) in view of Wivel et al (1993)

It is the Examiner's position that one skilled in the art at the time of filing the present application would have known that if germ-line modifications had applicability in humans, it would have had applicability in non-human animals because studies accomplished in humans are based on studies carried out in animals. The Appellants believe this rejection should not stand.

Wivel et al also relates to a different field making the combination of three pieces of art a combination from three different fields which clearly stretches the scope of what would reasonably have been consulted by the skilled artisan. Wivel et al relates to the introduction of foreign DNA into the pronucleus of a zygote or into a four or eight-cell embryo or the use of embryonic stem cells. There is no reference at all to male germ cell modifications. In fact, on page 533, column 3, about half-way down, notes:

> "It must be acknowledged, however, that at present most of the experimental work involves DNA transfer into one of the pronuclei of the zygote, the delivery of DNA into a four or eight-cell embryo by a vector, or the use of embryonic stem cells".

Given the above, the Appellants believe that the further combination of Wivel et al with Brinster et al and Vogel et al would not suggest the invention claimed in claim 160.

Appellants believe that the references have been compiled through hindsight and would not have been consulted in combination by anyone working in the art. In addition, at best, the references might suggest an approach to try and it appears that the Examiner has offered conclusions based on the

references that the references themselves do not suggest in order to arrive at the present rejections.

It remains that the Appellants believe that the combination of references cited and the present rejections is not proper but, in any event, they believe their claims to represent a clear inventive step over that combination.

CONCLUSION

Appellants believe that the Examiner has not sustained the burden of establishing a *prima facie* case of obviousness, and, therefore, the rejections based on 35 U.S.C. § 103 should not stand.

Appellants are convinced that the present claims are patentable and it is respectfully requested that the final rejection of the Examiner be reversed and the claims be allowed.

Respectfully submitted,

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United States Patent and Trademark Office

Assignments on the Web > Patent Query

Patent Assignment Abstract of Title

NOTE:Results display only for issued patents and published applications. For pending or abandoned applications please consult USPTO staff.

Total Assignments: 17

Patent #: 6316692 **Issue Dt:** 11/13/2001 **Application #:** 09191920 **Filing Dt:** 11/13/1998

Inventors: CAROL W. READHEAD, ROBERT WINSTON

Title: TRANSFECTION, STORAGE AND TRANSFER OF MALE GERM CELLS FOR GENERATION OF TRANSGENIC SPECIES & GENETIC THERAPIES

Assignment: 1

Reel/Frame: 009599/0661 **Recorded:** 11/13/1998 **Pages:** 3

Conveyance: ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

Assignor: READHEAD, CAROL W. **Exec Dt:** 01/08/1998

Assignee: CEDAR-SINAI MEDICAL CENTER
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Assignment: 2

Reel/Frame: 009599/0632 **Recorded:** 11/13/1998 **Pages:** 4

Conveyance: ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

Assignor: WINSTON, ROBERT **Exec Dt:** 01/23/1998

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Assignment: 3

Reel/Frame: 015108/0726 **Recorded:** 03/22/2004 **Pages:** 6

Conveyance: ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

Assignor: HOVATTA, OUTI **Exec Dt:** 03/03/2003

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Assignment: 4

Reel/Frame: 015320/0238 **Recorded:** 05/10/2004 **Pages:** 8

Conveyance: QUITCLAIM

Assignors: WINSTON, PROFESSOR LORD ROBERT MAURICE **Exec Dt:** 03/31/2004

HAMMERSMITH HOSPITAL NHS TRUST **Exec Dt:** 03/31/2004

Assignee: IMPERIAL COLLEGE INNOVATIONS LTD
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Assignment: 5

Reel/Frame: 015320/0246 **Recorded:** 05/17/2004 **Pages:** 8

Conveyance: ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

Assignor: IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE **Exec Dt:** 03/31/2004

Assignee: IMPERIAL COLLEGE INNOVATIONS LTD
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Assignment: 6

Reel/Frame: 015320/0267 **Recorded:** 05/17/2004 **Pages:** 5

Conveyance: ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

Assignor: IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE **Exec Dt:** 03/31/2004

Assignee: IMPERIAL COLLEGE INNOVATIONS LTD.
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Assignment: 7

Reel/Frame: 015361/0605 **Recorded:** 05/24/2004 **Pages:** 8

Conveyance: ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

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APPENDIX B TO APPELLANTS' BRIEF

APPEALED CLAIMS

The following represents the current status of all the claims in the present application including changes made by this paper:

1-134(canceled).

135(previously presented). An in vitro method of incorporating at least one polynucleotide encoding a desired trait into a male germ cell, comprising:

(a) obtaining a male germ cell from a non-human vertebrate species, said germ cell being selected from the group consisting of spermatogonial stem cells, type B spermatogonia, primary spermatocytes, preleptotene spermatocytes, leptotene spermatocytes, zygotene spermatocytes, pachytene spermatocytes, secondary spermatocytes, spermatids, and spermatozoa;

(b) transfecting the germ cell in vitro with at least one polynucleotide encoding a gene product in operable linkage with a promoter comprised in a virus or virus-derived DNA, in the presence of a gene delivery mixture comprising at least one transfecting agent, and optionally a polynucleotide encoding a genetic selection

marker;

(c) allowing the polynucleotide encoding a gene product to be taken up by, and released into the germ cell; and

(d) selecting those cells in which the polynucleotide has incorporated into the genome of the germ cell.

136(canceled).

137(previously presented). The method of claim 135, wherein the male germ cell is selected from the group consisting of spermatogonial cells and other undifferentiated male germ cells.

138(previously presented). The method of claim 135, wherein the transfection is conducted under conditions of temperature of about 25°C to about 38°C.

139(canceled).

140(previously presented). The method of claim 135, wherein the viral vector is selected from the group consisting of retroviral vectors, adenoviral vectors, transferrin-polylysine enhanced adenoviral vectors, Moloney murine leukemia virus-derived vectors, mumps vectors, and virus-derived DNAs that enhance polynucleotide uptake by and release into the cytoplasm of germ cells or a mixture of any members of said group.

141(previously presented). The method of claim 140, wherein the retroviral vector is selected from the group consisting of lentiviral vectors.

142(previously presented). The method of claim 135, wherein the transfecting agent comprises an adenovirus vector having endosomal lytic activity, and the polynucleotide encoding a gene product is operatively linked to the vector.

143(previously presented). The method of claim 135, wherein the polynucleotide encoding a gene product is in the form of a complex with a viral vector.

144(canceled).

145(previously presented). The method of claim 135, wherein:

the transfecting agent further comprises an agent selected from the group consisting of a c-kit ligand and at least one genetic selection marker; and

the method further comprises isolating or selecting a male germ cell carrying at least one polynucleotide encoding a gene product at least one polynucleotide encoding a genetic selection marker, from a donor male vertebrate with the aid of the genetic selection marker.

146(previously presented). The method of claim 145, wherein the genetic selection marker comprises a gene

expressing a detectable product, driven by a promoter selected from the group consisting of c-kit promoters, b-Myb promoters, c-raf-1 promoters, ATM (ataxia-telangiectasia) promoters, RBM (ribosome binding motif) promoters, DAZ (deleted in azoospermia) promoters, XRCC-1 promoters, HSP 90 (heat shock gene) promoters, and FRMI (from fragile X site) promoters.

147(previously presented). The method of claim 135, wherein the non-human vertebrate is a mammal.

148(previously presented). The method of claim 147, wherein the mammal is selected from the group consisting of non-human primates and farm and marine mammals.

149(previously presented). The method of claim 135, wherein the polynucleotide encoding a gene product is derived from the same non-human vertebrate species as the germ cell.

150(previously presented). The method of claim 135, wherein the non-human vertebrate is selected from the group consisting of wild and domesticated vertebrates.

151(previously presented). The method of claim 135, wherein the polynucleotide encoding a gene product is derived from a non-human mammal selected from the group consisting of human and non-human primates, canines, felines, swines, farm mammals, pachyderms, marine mammals,

equines, murines, ovines and bovines, or from a bird selected from the group consisting of ducks, geese, turkeys and chickens.

152(previously presented). The method of claim 151, wherein the polynucleotide is derived from a human.

153(previously presented). The method of claim 135, wherein the promoter is a germ cell-specific promoter.

154(previously presented). The method of claim 135, wherein the polynucleotide encoding a genetic selection marker is operatively linked to a germ cell-specific promoter.

155(previously presented). The method of claim 145, wherein the polynucleotide encoding a genetic selection marker is operatively linked to a germ cell-specific promoter.

156(previously presented). The method of claim 135 including a further step of introducing transfected cells selected in step (d) into a testis of a male of a non-human vertebrate of the species from which said male germ cell was obtained, thereby producing injected males.

157(previously presented). The method of claim 156 wherein said transfected cells are injected into said testis via vasa efferentia.

158(previously presented). The method of claim 156

including a further step of breeding one or more of said injected males to one or more normal females to thereby produce transgenic non-human mammal progeny.

159(previously presented). A transgenic non-human mammal produced by the method of claim 158.

160(previously presented). The method of claim 135 wherein said at least one polynucleotide encoding a gene product in step (b) is one which is able to correct a gene disorder.

APPENDIX C TO APPELLANTS' BRIEF

EVIDENCE APPENDIX

Capecchi[1] (1989) Trends in Genetics, 5:70-76

Smithies[2] (1993) Trends in Genetics, 9:112-116

Kim et al[3] (1997) Mol. Reprod. Dev., 46:515-526

Bachiller[3] et al (1991) Mol. Reprod. Dev., 30:194-200

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[1] Cited by the Examiner in an Action dated April 6, 2005
[2] Included by Appellants in a paper dated and entered September 16, 2005
[3] Cited by an Examiner in an Office Action originally dated October 6, 2003; resent April 14, 2004

THE NEW MOUSE GENETICS: ALTERING *the* GENOME *by* GENE TARGETING

MARIO R. CAPECCHI

Gene targeting (homologous recombination between DNA sequences residing in the chromosome and newly introduced DNA sequences) in pluripotent, mouse embryo-derived stem (ES) cells promises to provide the means to generate mice of any desired genotype. This review describes some of the background and current advances of gene targeting in mouse ES cells.

Our ability to carry out a comprehensive genetic analysis of an organism becomes more limited and difficult as the complexity of the organism increases. The additional complexity in the analysis is both operational and interpretive in nature. In the case of the mouse, limitations imposed by the attainable mutation rates, the sizes of the available mouse colonies and the generation time of the mouse make it impractical to isolate mutations of a desired class. Instead, the existing collection of mouse mutations, which is remarkably large and varied, was obtained by relying principally on serendipity to expose visible variation in morphology and/or behaviour. As a consequence, because they are more difficult to detect, very few recessive lethal mutations are present in this collection.

Complex organisms are likely to have not only more genes than simple organisms but also more elaborate networks of interactions among those genes. As a result of this increase in gene interaction, mutations in many genes may yield similar phenotypes. Analyses of some of these mutations might not be informative. As an example, mutations in metabolic pathways are anticipated to have profound effects on such complex phenomena as development or learning. Yet, establishing this linkage might not increase our understanding of these phenomena. In fact, it was this latter argument that led some investigators to voice scepticism as to whether a genetic analysis of complex phenomena in complex organisms would be fruitful. In addition, as organisms increase in complexity, more effort is needed to analyse a given mutation. In the absence of advance knowledge of which mutations are worthy of detailed characterization, the input work needed to examine many mutations would not seem to be justified by the meagre expected output of informative data.

When applied to the mouse, this argument was compelling. Recently however, the premise on which the above argument rests has completely changed. We are no longer dependent on random mutagenesis to generate mutations. Through gene targeting, the potential now exists to generate mice of any desired genotype. The experimenter chooses both which gene to mutate and how to mutate it. The criteria for selecting which gene to mutate can be based on knowledge generated within the species or from other species. The ability to choose how to mutate the gene will permit a thorough analysis of the function of any cloned gene through the generation of multiple mutant alleles. Not only can gene targeting be used to generate null alleles, it can be used to modify any property of the gene that affects its function, such as its transcriptional pattern, its mRNA or protein maturation pattern, or the ability of its protein product to interact with other gene products. It is hoped that the precision afforded by gene targeting will allow the formulation of genetic questions with sufficient clarity to yield informative answers.

The procedure for using gene targeting to generate mice with specific mutations first involves the use of standard recombinant DNA technology to introduce the desired mutation into a cloned DNA sequence of a chosen locus. That mutation is then transferred via homologous recombination to the genome of a pluripotent, embryo-derived stem (ES) cell[1,2]. Microinjection of the mutant ES cell into mouse blastocysts is used to generate germ-line chimeras[3]. Finally, interbreeding of heterozygous siblings yields animals homozygous for the desired mutation.

The application of this approach to mouse genetics is dependent on the availability of a cloned, genomic fragment of the chosen locus. At present this does not appear to be a limitation. The number of available cloned mouse genes that now exist is very large and new methods for isolating additional genes are continually being developed. A particularly fruitful method for identifying new mouse genes exploits the use of DNA sequences that are conserved between related genes from other species. Indeed, the number of available cloned mouse genes is growing extremely rapidly, whereas a functional assignment of these genes is grossly lagging. This situation will be even further exacerbated by the anticipated sequencing of the human and mouse genomes.

HOMOLOGOUS RECOMBINATION IN CULTURED MAMMALIAN CELLS

The ability to target exogenous DNA to specific chromosomal sites is dependent on the cells' intrinsic ability to mediate recombination between homologous DNA sequences. Our laboratory first demonstrated that mammalian somatic cells possess the enzymatic machinery for efficiently mediating homologous recombination between newly introduced, nonreplicating DNA molecules[4]. The analysis of the mechanisms of recombination between co-introduced, exogenous DNA sequences has become an active field of research[5–13]. From these studies it is evident that the cellular recombination machinery can mediate both nonconservative and conservative homologous recombination[10,12,13]: that is, formation of the products of homologous recombination may or may not result in the loss of input DNA sequences. Both reciprocal and nonreciprocal homologous recombination are possible, although there is a distinct preference for nonreciprocal reactions[11,14].

Given a choice, the machinery exhibits distinct preferences for transferring certain alterations, such as

small deletions or insertions, rather than other alterations such as point mutations[14]. The frequency of recombination between co-introduced DNA molecules is roughly proportional to the extent of homology between them. When DNA molecules share more than 5 kilobases of homology, then nearly every molecule introduced into the cell nucleus participates in at least one recombination event[4]. Recombination between co-introduced DNA molecules can, however, be detected between molecules sharing as little as 25–50 base pairs of homology[15]. DNA ends stimulate the reaction[4,9]. The ability to mediate homologous recombination is dependent on the cell's position in the cell cycle, showing a peak of activity in early S phase[16]. Kinetic analysis of homologous recombination between co-introduced DNA molecules indicates that the reaction occurs very rapidly, within 30 minutes after the DNA is introduced into the nucleus[11]. At later times, the DNA molecules become refractory to participation in homologous recombination. Exclusion from participation in homologous recombination coincides with the time at which the newly introduced DNA is packaged into chromatin.

These studies suggested that the cellular machinery could be exploited to mediate homologous recombination between newly added, exogenous DNA sequences and cognate chromosomal sequences. This reaction has been termed 'gene targeting'. Furthermore, these studies also suggested which parameters might influence the frequency of gene targeting.

Early gene targeting experiments

Although mammalian cells can efficiently mediate recombination between homologous DNA sequences, they demonstrate an even greater propensity for mediating nonhomologous recombination[17,18]. The problem is thus to identify homologous recombination events in a vast pool of scattered, nonhomologous recombination events. For this reason, early gene targeting experiments were based on cell lines carrying a mutated form of either a neomycin resistance (neo^r) gene or a herpes simplex virus thymidine kinase (HSV-tk) gene, integrated randomly in the host genome. That gene was then specifically repaired by homologous recombination with newly introduced plasmid DNA that carried the same gene bearing a different mutation, productive gene targeting events being identified by selection for cells with the wild type phenotype, either resistance to the drug G418 (neo^r) or ability to grow in HAT medium (tk^+)[19–22]. The advantages of these systems for analysing gene targeting are the ability to select directly for the targeting events and the ease of manipulating both the target and the incoming, exogenous DNA sequences.

Studies using these systems yielded a number of unexpected results.

(1) The frequency of targeting events was much higher than anticipated[14].

(2) Neither increasing the number of copies of the incoming plasmid DNA nor increasing the number of target sequences residing in the host genome increased the targeting frequency. This result suggested that the initial step in the targeting reaction, that of finding the homologous sequence in the genome, might not be the rate-limiting step. This was not anticipated since the incoming DNA has to search 3×10^9 bp of genomic DNA. A practical outcome of this result is that gene targeting experiments can be done using just a few molecules per cell. Under these conditions the probability that both homologous and nonhomologous recombination occurs in the same cell is small.

(3) The position of the target gene within the chromosome did not strongly influence the frequency of homologous recombination[14]. A dozen independent recipient cell lines, each containing the defective neo^r gene at a different chromosomal position, exhibited similar gene targeting frequencies. This observation suggested that a rather large proportion of the genome might be accessible to the recombination machinery.

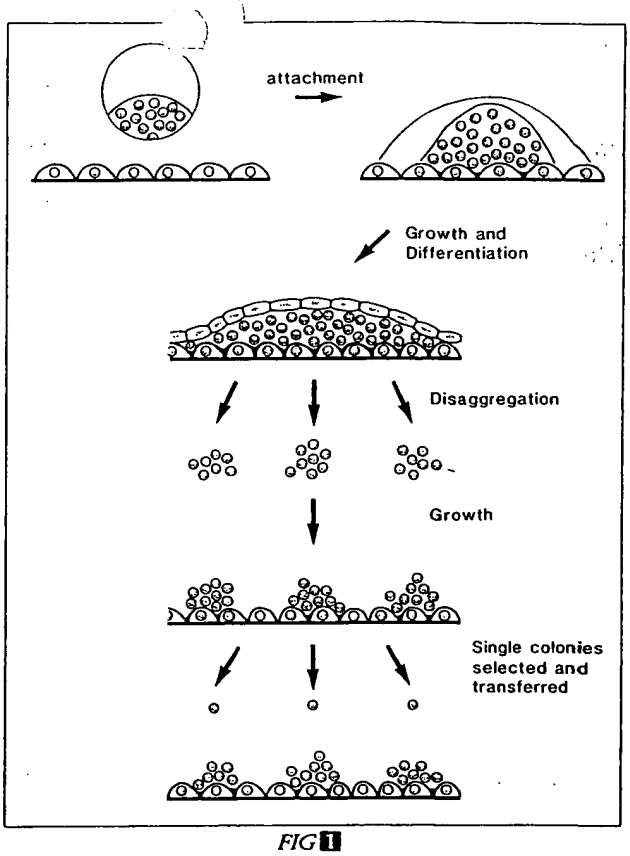

FIG 1

ISOLATION OF EMBRYO-DERIVED STEM (ES) CELLS FROM MOUSE BLASTOCYSTS[25,26]. A 4.5 DAY PREIMPLANTATION EMBRYO, THE BLASTOCYST, IS PLACED ON A MONOLAYER OF CELLS WHICH PROVIDE A MATRIX FOR ATTACHMENT AS WELL AS SECRETED PROTEIN FACTOR(S) WHOSE ROLE IS TO MAINTAIN THE CELLS' PLURIPOTENT CAPACITY BY INHIBITING NEWLY BORN ES CELLS FROM DIFFERENTIATION. FOLLOWING ATTACHMENT OF THE BLASTOCYST, THE CELLS IN THE INNER CELL MASS PROLIFERATE. AT THE APPROPRIATE TIME THE INNER CELL MASS IS PHYSICALLY REMOVED WITH A MICROPIPETTE, DISPERSED INTO SMALL CLUMPS OF CELLS, AND SEEDED ONTO NEW FEEDER CELLS. COLONIES ARE MICROSCOPICALLY EXAMINED FOR THE CHARACTERISTIC MORPHOLOGY OF ES CELLS. SUCH COLONIES ARE PICKED, DISPERSED INTO SINGLE CELLS AND RESEEDED ONTO FEEDER CELLS. IF THE PROCEDURE PROGRESSES SUCCESSFULLY, THESE CELLS YIELD COLONIES WITH A UNIFORM MORPHOLOGY, CHARACTERISTIC OF ES CELLS. THE ES CELLS CAN THEN BE TESTED FOR PLURIPOTENCY BY MEASURING THEIR CAPACITY TO DIFFERENTIATE *IN VITRO* AS WELL AS THEIR CAPACITY TO GENERATE GERM-LINE CHIMERAS.

(4) The input DNA could either transfer information to the target site precisely via homologous recombination or induce mutations in the target, a process we termed 'heteroduplex-induced mutagenesis'[19,23]. Fortunately, the configuration of the heteroduplex between the input DNA and the cognate chromosomal sequence needed to trigger induction of mutations is very restricted. Therefore, unless the appropriate conditions are set up deliberately, heteroduplex-induced mutagenesis is not observed.

The repertoire of gene targeting experiments was extended by Oliver Smithies and his colleagues, who demonstrated that an endogenous gene, the β-globin gene, was a suitable target[24]. Since insertion into the β-globin locus did not confer a selectable, cellular phenotype, Smithies *et al.* devised an elegant screen for identifying the predicted results of a targeting event using a λ library prepared from pools of transfected colonies. Armed with this assay, they then used sib selection to identify the original cell line in which the targeting event had occurred.

ES CELLS

In the early 1980s Evans and Kaufman[25], and Martin[26] isolated pluripotent, embryonic stem (ES) cells directly from mouse blastocysts. Their procedure is outlined in Fig. 2. When ES cells are reintroduced into a blastocyst, they contribute efficiently to the formation of all tissues in a chimeric mouse, including the germ line[3]. It has also been shown that ES cells can be manipulated *in vitro* without losing their capacity to generate germ-line chimeras. For example, after transfection with the neo^r gene and selection for G418-resistant cells, the selected cells were used to generate germ-line chimeras[27,28]. In addition, mice deficient in hypoxanthine phosphoribosyl transferase (hprt) were produced from ES cells that were either selected for spontaneous *hprt*$^-$ mutations[29] or selected for *hprt*$^-$ after random insertion of retroviral DNA into the ES cell genome[30].

The procedure for generating germ-line chimeras from ES cells is outlined in Fig. 2. If the coat colour alleles of the donor ES cells and the recipient blastocyst are distinguishable, the resulting chimeric mouse will exhibit coat colour mosaicism. Whether donor ES cells contribute to the germ line can then be evaluated by breeding to a suitable mouse.

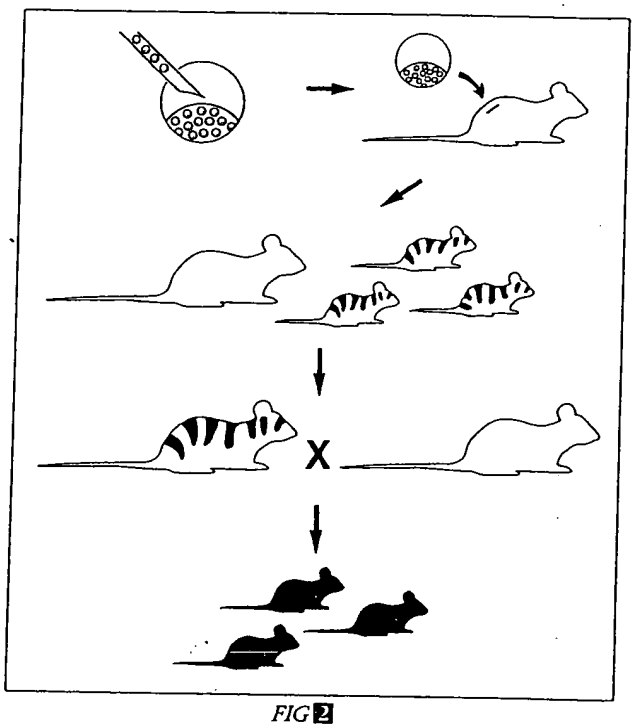

FIG 2

GENERATION OF MOUSE GERM-LINE CHIMERAS BY INTRODUCING ES CELLS INTO MOUSE BLASTOCYSTS[3]. FIRST, ES CELLS ARE MICROINJECTED INTO THE BLASTOCOEL CAVITY OF A 4.5 DAY EMBRYO. THE BLASTOCYST IS THEN SURGICALLY TRANSFERRED INTO THE UTERUS OF A PSEUDOPREGNANT MOUSE AND DEVELOPMENT IS ALLOWED TO PROGRESS TO BIRTH. ABOVE, THE ES CELLS WERE DERIVED FROM A MOUSE HOMOZYGOUS FOR THE BLACK COAT COLOR ALLELE AND THE RECIPIENT BLASTOCYST FROM A MOUSE HOMOZYGOUS FOR THE ALBINO ALLELE. THE CHIMERIC MICE ARE COMPOSED OF CELLS OF BOTH GENOTYPES AND THEREFORE DISPLAY COATS WITH PATCHES OF EACH COLOR. BREEDING OF A CHIMERIC MOUSE TO AN ALBINO MOUSE YIELDED BLACK MICE, FROM WHICH WE CAN CONCLUDE THAT THE ES CELLS CONTRIBUTED TO THE FORMATION OF THE GERM LINE.

TARGETED DISRUPTION OF THE *hprt* GENE

The ability to modify specific, chosen sites of the ES cell genome by gene targeting clearly provided the potential for generating mice of any desired genotype. Towards achieving this goal, we set out to inactivate the endogenous *hprt* gene in ES cells. Because this gene is on the X chromosome, only one mutant copy was needed to yield the recessive *hprt*$^-$ phenotype in male ES cells. Furthermore, *hprt*$^-$ cells could be directly selected by growth in the presence of the cytotoxic base analogue 6-thioguanine (6-TG). The results from these experiments established the feasibility of gene targeting in ES cells and defined some of the parameters that control the efficiency of this process[31].

Figure 3 illustrates the targeting vectors used to disrupt the *hprt* gene. Two classes of vectors were tested: sequence replacement and sequence insertion vectors. As the names imply, sequence replacement vectors replace endogenous DNA with exogenous sequences, whereas sequence insertion vectors insert the entire vector DNA sequence into the endogenous locus. In each class of vectors, the eighth exon of *hprt* is disrupted by the insertion of the neo^r gene, which both disrupts the coding sequence of *hprt* and acts as a selectable marker for cells containing an integrated copy of the recombinant vector.

After the targeting vector was introduced into ES cells by electroporation and selection for G418^r/6-TG^r cells, all survivors were found to have lost *hprt* activity as a result of a targeted disruption of the *hprt* gene. Interestingly, replacement vectors and insertion vectors were equally efficient in disrupting the endogenous *hprt* gene. Furthermore, both vectors showed the same dependency of the targeting frequency on the extent of homology between the exogenous and endogenous DNA sequences[31]. A twofold increase in homology resulted in a 20-fold increase in the targeting frequency.

Smithies and his colleagues have also reported targeting to the *hprt* locus in ES cells[32]. In their experiments, the recipient ES cell line contained a 5'-deletion

in the *hprt* gene[29] and they corrected the mutation by homologous recombination with a sequence insertion vector. The absolute frequency of their targeting events was comparable to those described above.

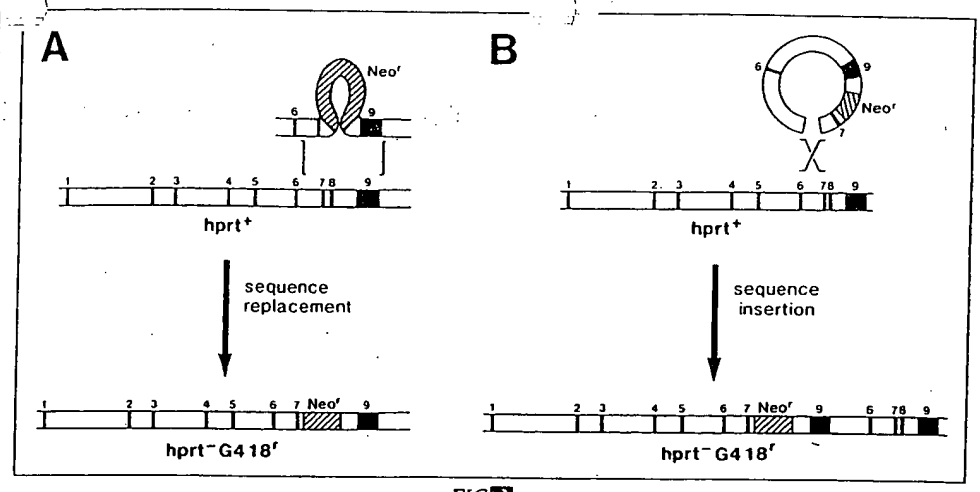

FIG 3

DISRUPTION OF THE *hprt* GENE BY GENE TARGETING USING (A) A SEQUENCE REPLACEMENT TARGETING VECTOR OR (B) A SEQUENCE INSERTION TARGETING VECTOR. VECTORS OF BOTH CLASSES CONTAIN *hprt* SEQUENCES INTERRUPTED IN THE EIGHTH EXON WITH THE neo^r GENE. WITH THE SEQUENCE REPLACEMENT VECTOR, FOLLOWING HOMOLOGOUS PAIRING BETWEEN THE VECTOR AND GENOMIC SEQUENCES, A RECOMBINATION EVENT REPLACES THE GENOMIC SEQUENCE WITH VECTOR SEQUENCES CONTAINING THE neo^r GENE. SEQUENCE INSERTION VECTORS ARE DESIGNED SUCH THAT THE ENDS OF THE LINEARIZED VECTOR LIE ADJACENT TO ONE ANOTHER ON THE *hprt* MAP. PAIRING OF THESE VECTORS WITH THEIR GENOMIC HOMOLOG, FOLLOWED BY RECOMBINATION AT THE DOUBLE STRAND BREAK, RESULTS IN THE ENTIRE VECTOR BEING INSERTED INTO THE ENDOGENOUS GENE. THIS PRODUCES A DUPLICATION OF A PORTION OF THE *hprt* GENE.

GENERATING OTHER TYPES OF MUTATIONS

Insertion of the neo^r gene into the coding region of a target gene creates a null mutation. A similar strategy can be used to generate other types of mutations. For example, if the neo^r gene is located in an intron or 3' untranslated exon within the targeting vector, the neo^r gene still provides a tag for the input DNA but does not disrupt the coding sequence of the target gene. Any desired mutation – an insertion, deletion or substitution – can then be placed anywhere in the targeting vector.

Since with sequence replacement vectors the positions of the crossovers between the targeting vector and the target sequence are not specified, transfer of the desired mutation with the selectable gene relies on co-conversion. Surprisingly, this co-conversion frequency is very high. Small nucleotide substitutions as far as 3 kilobases from the neo^r gene are co-converted with neo^r at a frequency of 88% (K. Thomas and M. Capecchi, unpublished). With sequence insertion vectors the entire targeting vector is inserted into the target, thereby automatically transferring both the desired mutation and the selectable gene into the endogenous locus.

NONSELECTABLE GENES

The *hprt* gene was chosen as a target because one could directly select for cells in which a targeting event had occurred. However, most genes are present as two copies in the genome, and in the vast majority of cases a selectable cellular phenotype is not associated with the inactivation of either one or both copies of the gene. Therefore, isolation of the rare ES cell in which a nonselectable gene has been inactivated can only be achieved by using an indirect enrichment and/or screening procedure.

One of the most sensitive screening procedures involves the use of the polymerase chain reaction (PCR)[33] to detect a predictable, novel DNA junction created by the targeting event[34]. The use of PCR to detect such a junction is illustrated in Fig. 4. PCR can be used to detect the results of targeted disruptions if the absolute frequency of such events is greater than one event in 1000–10 000 starting cells. At present it is difficult to predict how much the targeting frequency will vary from locus to locus. In particular, the frequency of disrupting a gene that is not expressed in ES cells remains unknown.

An alternative approach is to couple screening and enrichment procedures. If the target locus is transcribed in ES cells and the locations of the *cis*-acting transcriptional signals of the target gene are known, then an enrichment procedure can be used that takes advantage of activating a defective, selectable gene. For example, a neo^r gene lacking an enhancer or a promoter can be positioned within the targeting vector such that homologous recombination with the target gene would juxtapose the defective neo^r gene with the sequences required for effective expression. Random integration of the same vector into the recipient genome would not normally bring the required activating sequences sufficiently near the defective neo^r gene to yield G418^r colonies. Jasin and Berg have shown that such procedures can yield several hundred-fold enrichment for cells in which a targeting event has occurred[35].

Recently, we have described an enrichment procedure that is independent of the function of the gene and may be applicable to genes that are not expressed in ES cells[36]. The intron–exon boundaries within the cloned fragment of the target gene must be known but no other information is required. The procedure uses a positive selection for cells that have incorporated the target vector anywhere in the ES genome and a negative selection against cells that have randomly integrated the vector at sites other than the target. The net effect of this positive–negative selection is to enrich for cells containing the desired targeted mutation.

This procedure was used to isolate ES cells containing targeted disruptions in the *hprt* and *int-2*

genes[36]. The *int-2* protc') gene was first identified as a gene activated in mammary tumours of mice by the nearby insertion of the mouse mammary tumour virus[37]. The *int-2* gene codes for a protein with significant sequence similarity to the fibroblast growth factor family. *In situ* hybridization analysis of mouse embryo sections has revealed diverse but highly restricted patterns of expressions suggesting that *int-2* may have several different functions during embryogenesis. To define these roles a genetic analysis of *int-2* was initiated.

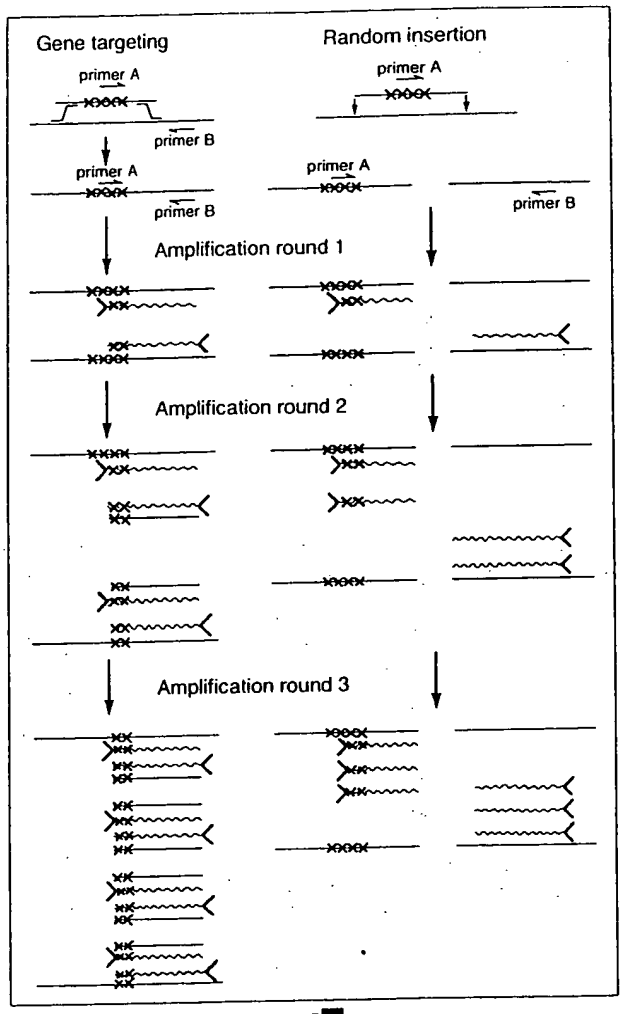

FIG 1

DETECTION OF A RECOMBINANT FRAGMENT USING POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION. ONE DNA POLYMERASE PRIMER IS CHOSEN TO HYBRIDIZE UNIQUELY TO SEQUENCES ON THE INCOMING, TARGETING VECTOR (PRIMER A), THE OTHER PRIMER WITH SEQUENCES ON THE OPPOSITE DNA STRAND IN THE ENDOGENOUS TARGET GENE (PRIMER B). IF THERE IS HOMOLOGOUS RECOMBINATION BETWEEN THE INCOMING DNA AND THE TARGET SEQUENCE (LEFT SIDE OF THE FIGURE), THE TWO PRIMER HYBRIDIZATION SITES WILL BE JUXTAPOSED AND PCR WILL EXPONENTIALLY AMPLIFY THE INTERVENING DNA. AFTER 20 ROUNDS OF AMPLIFICATION THE INTERVENING DNA WILL BE AMPLIFIED APPROXIMATELY A MILLION-FOLD (2^{20}). IF THERE IS RANDOM INSERTION OF THE TARGETING VECTOR INTO THE HOST GENOME (RIGHT SIDE OF FIGURE), THE TWO PRIMER HYBRIDIZATION SITES WILL BE ON SEPARATE PIECES OF DNA AND PCR WILL LINEARLY AMPLIFY THE DNA FROM PRIMER A AND B (I.E., AFTER 20 ROUNDS OF PCR THE DNA SYNTHESIZED FROM PRIMER A AND B WILL BE AMPLIFIED 20-FOLD).

POSITIVE–NEGA' ' ECTION (PNS)

Figure 5 o' ies the experimental approach used to enrich for those rare cells in which the exogenous DNA recombined with its cognate chromosomal sequence. The recombinant vector, of the replacement class, contains 10–15 kb of DNA homologous to the target gene, X; a neo^r gene inserted into an exon of that sequence; and an HSV-tk gene adjacent to the sequence.

This vector was designed so that when the endogenous X sequence is replaced by the exogenous DNA via homologous recombination, the HSV-tk gene will not be transferred into the target gene (Fig. 5A). Exclusion of the HSV-tk gene during homologous recombination occurs because the HSV-tk gene represents a discontinuity in the incoming vector between homology and nonhomology with the endogenous target sequence. Cell lines in which the targeting event occurs will therefore be X$^-$, neo^r , HSV-tk$^-$. On the other hand, random integration of the targeting vector into the recipient cell genome should, in most cases, result in cells that are X$^+$, neo^r, HSV-tk$^+$ (Fig. 5B). This is derived from the observation that we and others have made that most random insertions of exogenous, linearized DNA into the genome occur via their ends[4,14,18]. Therefore, by selecting for cells containing a functional neo^r gene (G418^r) and against cells containing a functional HSV-tk gene (gancyclovir resistance, GANC^r), we enrich for cells in which the targeting event has occurred.

This positive–negative selection (PNS) procedure yielded a 2000-fold enrichment for ES cells containing targeted disruptions of the *hprt* and *int-2* genes over the total number of neo^r cells[36]. Since introduction of the *hprt* targeting vector into ES cells yielded approximately one targeted event for every 2000 random insertion events when there was no indirect selection for targeting, virtually every G418^r-GANC^r ES cell line that we obtained (19/24) contained a targeted disruption of the *hprt* gene, even though we did not directly select for the *hprt*$^-$ phenotype.

The targeting frequency into the *int-2* locus was 20-fold lower, one in 20 G418^r-GANC^r cell lines containing a disrupted *int-2* gene. This lower frequency of targeting may reflect the lower level of *int-2* expression in ES cells: whereas *hprt* transcripts can be readily detected in ES cells, *int-2* is transcribed at a level of less than one transcript per cell[36]. The PNS procedure was also used to create null mutations in the mouse homeobox genes *box 1.2* and *box 1.3* (D. Kostic and M. Capecchi, unpublished). Like *hprt*, *box 1.2* and *box 1.3* are expressed at moderate levels in ES cells and exhibit similar gene targeting disruption frequencies.

TARGETING INTO GENES NOT EXPRESSED IN ES CELLS

Targeted disruptions of genes not expressed in ES cells have not been reported. Preliminary attempts to disrupt one such gene, the proto-oncogene *int-1*, indicate a lower targeting frequency than for *hprt*, *int-2*, *box 1.2* or *box 1.3* (K. Thomas and M. Capecchi, unpublished). For genes that are recalcitrant to targeted disruption, one may be able to increase the enrich-

ment factor of the PNS procedure an additional 2000-fold by inserting two HSV-tk genes into the targeting vector, one at each end; at least one HSV-tk gene should then survive the transfection procedure unmutated to permit selection against cells that contain random integrations of the targeting vector. If one HSV-tk gene acquires a deleterious mutation during transfection at a frequency of one in 2×10^3 molecules, then the frequency of inactivation of both HSV-tk genes should be one in 4×10^6 molecules. (Surprisingly, placing a large block of nonhomology at both ends of the linearized targeting vector does not hinder participation in homologous recombination; K. Thomas and M. Capecchi, unpublished.)

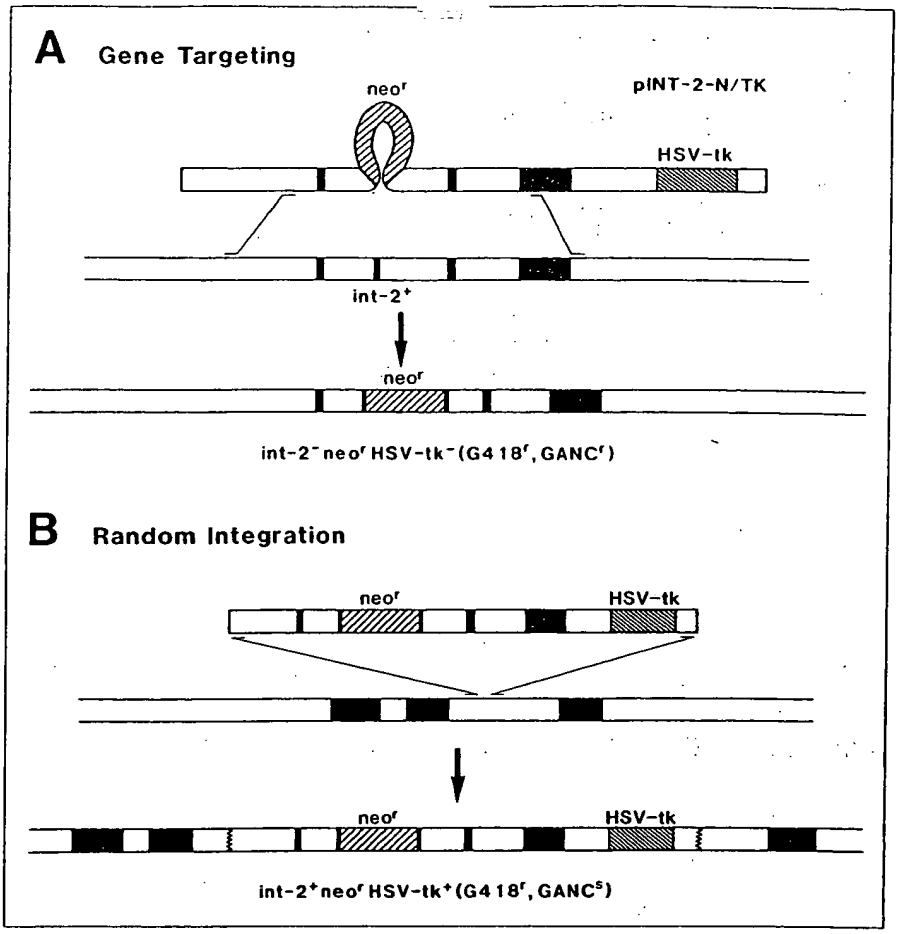

FIG 5

THE POSITIVE–NEGATIVE SELECTION (PNS) PROCEDURE USED TO ENRICH FOR ES CELLS CONTAINING A TARGETED DISRUPTION OF GENE X. (A) A GENE X SEQUENCE REPLACEMENT VECTOR THAT CONTAINS AN INSERTION OF THE neo^r GENE IN AN EXON OF GENE X AND A LINKED HSV-TK GENE IS SHOWN PAIRING WITH A CHROMOSOMAL COPY OF GENE X. HOMOLOGOUS RECOMBINATION BETWEEN THE TARGETING VECTOR AND GENOMIC X DNA RESULTS IN THE DISRUPTION OF ONE COPY OF GENE X AND THE LOSS OF HSV-TK SEQUENCES. SUCH CELLS WILL BE X^- neo^r AND HSV-TK^- AND WILL BE RESISTANT TO BOTH G418 AND GANC. (B) BECAUSE NONHOMOLOGOUS INSERTION OF EXOGENOUS DNA INTO THE GENOME OCCURS THROUGH THE ENDS OF THE LINEARIZED DNA, THE HSV-TK GENE REMAINS LINKED TO THE neo^r GENE. SUCH CELLS WILL BE X^+, neo^r, HSV-TK^+ AND THEREFORE RESISTANT TO G418 BUT SENSITIVE TO GANC. GANCYCLOVIR IS A NUCLEOSIDE ANALOGUE THAT SPECIFICALLY KILLS CELLS THAT CONTAIN A FUNCTIONAL HSV-TK GENE AND IS NOT CYTOTOXIC TO CELLS CONTAINING THE CELLULAR TK GENE[36].

USES OF GENE TARGETING

Generation of specific mouse mutants via gene targeting should greatly facilitate the analysis of all phases of mouse biology including development, cancer, immunology and neurobiology. Recent molecular genetic analysis of development in *Drosophila* has revealed a network of genes that control its metameric pattern formation[38]. On the basis of DNA sequence similarity, related genes have been identified in the mouse[39,40]. The function of these mouse genes, however, is not known. The embryonic expression pattern of these genes implies a role in establishing positional information during development of the mouse, but how closely the function of the mouse genes parallels the function of the *Drosophila* homologues remains to be determined.

Very recently, one of these genes, *pax-1*, has been strongly correlated with a mouse mutation identified in 1947, *undulated*[41]. Homozygous mice show vertebral malformations along the entire head to tail axis, implying a role for *pax-1* in the generation of the vertebral column. Unfortunately, it has not been possible to correlate any of the other 40 or so mouse 'homeobox' or 'paired box' genes with known mouse mutations. Targeted disruptions of these genes may not only reveal the phenotypes associated with inactivation of the individual genes, but, through epistasis, and molecular analysis, may also help define the developmental network controlling mouse morphogenesis. Targeted disruptions of the mouse homeobox genes *box 1.1*, *box 1.2*, *box 1.3* and *en-2* have already been achieved (recently described at a Cold Spring Harbor meeting on the molecular genetics of the mouse, *box 1.1* by A. Zimmer and P. Gruss, *box 1.2* and *box 1.3* by D. Kostic and M. Capecchi and *en-2* by A. Joyner and J. Rossant).

Gene targeting can also be expected to contribute significantly to neurobiology, providing a rational and new approach to genetic analysis of the intimidatingly complex mammalian nervous system. As a representative mammal, the mouse should again profit from the molecular-genetic analysis of less complex organisms such as *C. elegans* and *Drosophila*. Many interesting genes that affect the functioning of the nervous system in these organisms are likely to be conserved in mammals. Gene targeting should allow the evaluation of the roles of such genes as well as the roles of genes

recently identified directly in the mouse on the basis of intriguing, highly neuron-specific patterns of expression. By combining this new genetic analysis with other already established analytical approaches, we might begin to achieve some understanding of a molecular mechanism underlying development of the mammalian nervous system as well as its most elusive functions such as learning and memory.

Of more immediate application to human medicine, targeted disruptions in the mouse should provide mouse models for human genetic diseases. Such models should prove useful for analysing the pathology of the disease as well as providing systems for exploration of new therapeutic protocols, including gene therapy. It will be interesting to see if gene targeting in human somatic cells can be directly applied to human gene therapy. Correction of the endogenous defective gene in the appropriate human tissue has obvious advantages over the random insertion of a nondefective gene: for example, the corrected endogenous gene is much more likely to be expressed at appropriate levels. As researchers become able to culture and propagate a variety of human somatic stem cells such as hematopoietic stem cells, epithelial stem cells, liver and lung stem cells, somatic gene therapy protocols may become possible based on gene targeting to correct the endogenous gene in the appropriate tissue.

Gene targeting may also provide an additional tool for mapping and/or isolating human genes. Gene targeting can be used to insert dominant selectable genes into specific chromosomal regions. Once the selectable gene is in place, it can be used to transfer that chromosomal region, via chromosome-mediated gene transfer, into new recipient cells, or to isolate deletions surrounding the selectable gene or to clone DNA sequences in its vicinity.

Dedication

As I write this article at the end of the year of his sixtieth birthday, I would like to dedicate the article to James D. Watson. Molecular biology, as we know it today, was born with the elucidation of the structure of DNA. Thirty-five years later we are on the brink of being able to change at will any nucleotide in the complex genome of a living mouse. On a more personal note, my own scientific career was born in Jim's laboratory. It is difficult in a few sentences to describe what Jim gave to each of us as we journied through his laboratory: on the one hand a sense of the invincible – if we fully applied ourselves we could wrestle from nature the solution to the most complex problem; on the other hand, a sense of awe, excitement and anticipation because her solution would be more elegant than we could ever imagine.

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1: Trends Genet. 1993 Apr;9(4):112-6.

Animal models of human genetic diseases.

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Gene targeting in cultured embryonic stem cells permits the generation of mice with a desired alteration in a chosen target gene. Application of this procedure to create mouse models of human diseases is revealing the innate complexity of diseases normally ascribed to single gene defects. Modeling human diseases that are known to be multigenic in origin and are markedly influenced by environmental factors is potentially even more revealing.

Publication Types:
- Review
- Review, Tutorial

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MOLECULAR REPRODUCTION AND DEVELOPMENT 46:515–526 (1997)

Development of a Positive Method for Male Stem Cell-Mediated Gene Transfer in Mouse and Pig

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ABSTRACT Classical approaches for producing transgenic livestock require labor-intensive, time-consuming, and expensive methods with low efficiency of transgenic production. A promising approach for producing transgenic animals by using male stem cells was recently reported by Brinster and Zimmermann (1994: Proc Natl Acad Sci 91:11298–11302) and by Brinster and Avarbock (1994: Proc Natl Acad Sci USA 91:11303–11307). However, in order to apply this technique to producing transgenic animals, some difficulties have to be overcome. These include a satisfactory method for short-term in vitro culture for drug selection after transfection with exogenous DNA, and methods for the use of livestock such as pigs. We developed a new method for transferring foreign DNA into male germ cells. Mice and pigs were treated with busulfan, an alkylating agent, to destroy the developing male germ cells, and liposome/bacterial LacZ gene complexes were introduced into each seminiferous tubule by using a microinjection needle. As a control, lipofectin was dissolved in phosphate-buffered saline at a ratio of 1:1, and then injected into seminiferous tubules. In mice, 8.0–14.8% of seminiferous tubule expressed the introduced LacZ gene, and 7–13% of epididymal spermatozoa were confirmed as having foreign DNA by polymerase chain reaction. The liposome-injected testes were all negative for X-gal staining. These results indicate that some spermatozoa were successfully transformed in their early stages by liposome/DNA complexes. In pigs, foreign DNA was also incorporated efficiently into male germ cells, and 15.3–25.1% of the seminiferous tubules containing germ cells expressed the LacZ gene. The data suggest that these techniques can be used as a powerful tool for producing transgenic livestock. *Mol. Reprod. Dev. 46:515–526, 1997.*

Key Words: transgenic; sperm vector; male stem cells; liposome; testis; mouse; pig; LacZ

INTRODUCTION

The first genetically engineered transgenic mice were produced by microinjecting foreign DNA into the pronucleus of a zygote, and then subsequently transferring the zygote into a recipient female (Palmiter et al., 1982; Gordon and Ruddle, 1983; Palmiter and Brinster, 1986; Brinster et al., 1993). Although a number of researchers have reported production of transgenic sheep (Hammer et al., 1985; Wright et al., 1991), pigs (Pursel et al., 1989), and cattle (Bowen et al., 1994; Strijker et al., 1992), progress with livestock has been hampered for several reasons. A fundamental problem in the production of transgenic livestock is a limited supply of early embryos at the proper stages of development. Furthermore, it is very expensive and laborious to obtain zygotes by superovulation and surgical collection, and this often results in an asynchronous population of embryos. In addition, both the survival of a zygote after microinjection and the integration frequency of exogenous genes in farm animals are very low (Eyestone, 1994). Therefore, the sperm vector as an alternative methodology has been developed (Arezzo, 1989; Atkinson et al., 1991; Lavitrano et al., 1989, 1992; Laura and Gandolfi, 1993; Zani et al., 1995), but this technique remains unproved and is still under examination (Al-Shawi et al., 1990; Brinster et al., 1989; Maddox, 1989). Even though several theoretical approaches, such as retroviral vectors (Kim et al., 1993) and embryonic stem cells (Anderson, 1992; Stewart, 1991), have been addressed, no group has produced transgenic livestock by using these methods.

Recently, it was reported that early male germ cells, before the onset of chromatin condensation, were successfully transfected and cultured in vitro to round spermatids having haploid chromosomes (Hoffman et al., 1992, 1994; Minoo et al., 1993; Plauis-Flucklinger et al., 1993). Brinster and Avarbock (1994a) and Brinster and Zimmermann (1994b) reported a technique with which they transplanted testis-derived cells into mouse seminiferous tubules of infertile recipients and produced progeny derived from donor male germ cells. However, the application of these two techniques to produce transgenic animals will depend upon the cul-

Abbreviations: X-gal, 5-brome-4-chloro-3-indolyl-D-galactoside; PCR, polymerase chain reaction; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; IVF, in vitro fertilization; PMSG, pregnant mare's serum gonadotropin; hCG, human chorionic gonadotropin; EDTA, ethylenediaminetetraacetic acid.

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(CIAP, Takara) and tested by ligation reaction. Spermatozoa transfected with a 5.89-kb *HindIII/EcoRI* fragment derived from pZIP(X)hEPOSVneo plasmid were investigated for an array of integrations by PCR. Primers for PCR were prepared according to the method proposed by Burdon and Wall (1992): for detection of head and tail (340 bp), anti 5'-CGTAAACTGAACCAAGATACG-3' (primer 1), and 5'-CAGGAGTGGGGAGGCACGAT-3' (primer 2); for detection of single copy (867 bp), 5'-GAGTTGGGAAGCTAGACACTG-3', and anti 5'-CTCTCCCTCCTGCCCCTCAGCAGC-3'. PCR of spermatozoa was performed according to the method described by Li et al. (1988): the spermatozoa were treated with DNase I, and then completely washed, boiled at 95°C for 10 min to extract DNA, and cooled rapidly for 3 min. At 15 hr after in vitro fertilization, zygotes were enucleated by drawing the male pronucleus into a 20-μm beveled pipet as described by Hogan et al. (1994). After enucleation, a male pronucleus was washed into the oocyte manipulation medium, boiled in PCR buffer at 100°C for 5 min, and analyzed by PCR as described above.

Busulfan Treatment

Mice and pigs were treated with busulfan, an alkylating agent, according to the previous methods with minor modifications (Bucci and Meistrich, 1987; Brinster and Zimmermann, 1994b). Busulfan (40–100 mg/kg of body weight) was completely dissolved using 50 μl of N, N-dimethy-formamide (Sigma), and then 50 μl of sesame oil were added. The solution was injected intraperitoneally once in mice or twice every week for 5 weeks in pigs. In this study, 28 mice and 8 pigs from age 8–12 weeks were used. Among them, 19 mice and 4 pigs were used for histological analysis following busulfan treatment. Nine mice and 4 pigs were used for gene transfer.

Transfer of Liposome/DNA Complex Into Seminiferous Tubules

The mouse testis was exposed by laparotomy. The tunica albuginea of both testes was pierced partially by using a sharp 30-gauge needle, and approximately 5–10 μl of liposome/bacterial LacZ gene, derived from pCH110 plasmid under the control of SV 40 promoter (Takeda and Toyoda, 1991), complexes (4 μg/1 μg) were introduced into a site of seminiferous using a microinjection needle under light microscopy (Fig. 3). In the pigs, 4 weeks after the first busulfan treatment, the testes were exposed by surgical operation. Then, approximately 500 μl of DNA/liposome (rate of 1:4; 10 μg/40 μg) complexes were randomly introduced into 3 or 4 sites of both testes using a glass microinjection needle after piercing the tunica albuginea with a 30-gauge needle. Mice and pigs were injected with the liposome/DNA complexes twice 7 days between treatments.

Separation of Male Germ Cells

Spermatogenic and spermiogenic cells were prepared from the testes and purified by the Celsep® (Eppendorf) separation system, according to the method described by Bellve (1993) with minor modifications. Briefly, 4–8 weeks after transfection, the tunica was removed and testes were dissociated by mincing with scissors. The cells were incubated for 30 min at 37°C in HBSS (58.44 mM NaCl, 74.55 mM KCl, 137.9 mM Na_2PO_4, 0.1% glucose, and 84.01 mM $NaHCO_3$) containing 1 mg/ml of collagenase type I (Sigma, catalogue no. C-0130) and 1 μg/ml of DNase I. After centrifugation, cells were resuspended with 0.25% trypsin/1 mM EDTA and incubated for 5 min at room temperature. Then, trypsin was removed by centrifugation, and cells were separated on a linear gradient of 2–4% Percoll in PBS. After 2 hr of sedimentation at 4°C, cells were collected as 40-ml fractions. The cell types of each fraction were examined under light microscopy, and fractions containing the same cell types were pooled, washed twice with PBS, and then analyzed for LacZ expression.

X-Gal Assay

Four to 8 weeks after transfection, testes were dissociated and fixed in 2% formaldehyde for at least 2 hr, and then stained overnight in solution (1.0 mg/ml of X-gal, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in PBS, (pH 7.4) (Vernet et al., 1993). The stained testes were fixed again, embedded in paraffin, and sectioned by microtome. Expression of the bacterial LacZ gene in testes was investigated under light microscopy. Spermatocytes and round/elongating spermatids separated by Celsep® or preimplantation embryos were fixed on fixation solution (2% formaldehyde and 0.2% glutaraldehyde in PBS, pH 7.4, without Mg^{+2} and Ca^{+2} for 10 min at 4°C). The germ cells were washed twice in PBS, and stained with X-gal for 12 hr.

Immunohistochemistry

Antibody against β-galactosidase was purchased from Oncogene Science. Tissue for immunostaining was cleared in histoclear for approximately 10 min and dehydrated in decreasing concentrations of ethanol. Immunohistochemistry was performed according to standard procedures provided by the manufacturer (Mouse, Rabbit and Rat UniTect® Immunohistochemistry System, Oncogene Science). Sections were placed in 3% peroxide in pure methanol and 0.1% of pepsin in 0.05 N HCl, pH 2.25, for 30 min to reduce background staining. Sections were washed twice (5 min each) in TBS (0.05 M Tris-HCl, pH 7.4, and 0.85% NaCl) and blocked with normal horse serum diluted in TBS (1:5; NSS-TBS). Sections were incubated for 30 min with primary β-galactosidase antibody diluted at a concentration of 1:500 in NHS:TBS. One drop of horse serum from the ABC Kit was used as a negative control. Excess antibody was removed by washing twice for 5 min with TBS, and then biotinylated secondary IgG was added for 30 min, with a rinsing with 3 changes of TBS for 5 min. Sections were incubated with ABC reagent for 30 min and washed extensively with TBS, and rinsed in 1% Triton-X-PBS for 30 sec. The color

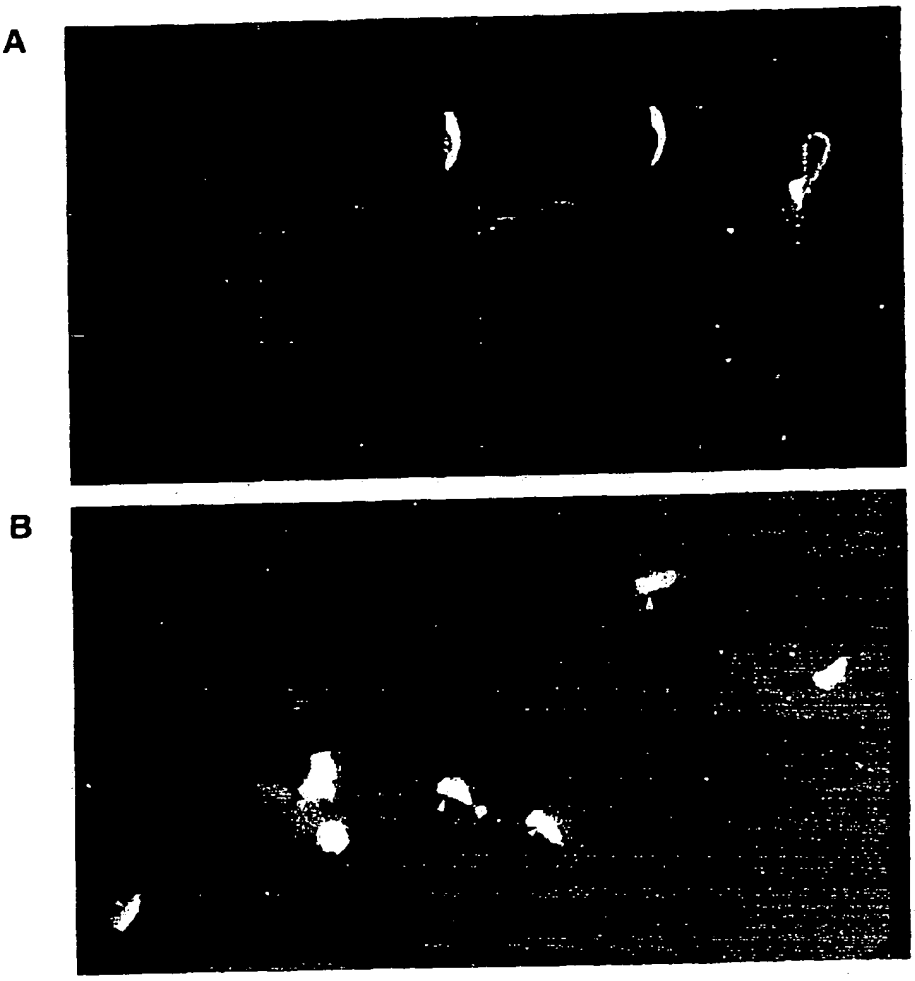

Fig. 2. Association of exogenous DNA in spermatozoa examined by fluorescent in situ hybridization. The signal of the exogenous DNA was detected as a green fluorescence of the FLUOS-conjugated anti-digoxigenin antibody by epifluorescence microscopy. A and B: Patterns of exogenous DNA binding in sperm cells. Yellow represents a complementary color between green and red. Yellow or green (arrow) indicates sperm cells labeled with foreign DNA.

by the transfected spermatozoa were examined for the presence of exogenous DNA. By PCR, exogenous DNA was not detected in enucleated nuclei (0/20), but was occasionally detected in the cytoplasm (2/30). These data indicate that liposome/DNA complexes can be bound onto spermatozoa efficiently, but cannot be incorporated into their chromosome DNA.

In Vivo Transfection of Male Stem Cells

Using sections of mouse testes, we investigated the effects of busulfan in the destruction of developing spermatocytes after treatment with busulfan. At 4 weeks after treatment with busulfan, only type A spermatogonia and a few developing spermatocytes in seminiferous tubules remained. At 12 weeks, the remaining stem cells were actively dividing and they showed a similar spermiogenesis and spermatogenesis (data not shown). To obtain efficient stem-cell transfection, male mice were pretreated with busulfan. Three weeks after injection with busulfan, liposome/DNA complexes were directly transferred into mouse seminiferous tubules (Fig. 3). Five of the 9 mice lost body weight severely and died within 2 weeks of transfer. However, testes from both dead and living mice efficiently expressed the bacterial LacZ gene in spermatogonia and developing spermatocyte cells, as well as in Leydig and Sertoli cells (Figs. 4, 5). Furthermore, the bacterial LacZ gene was broadly expressed in spite of partial transfer of foreign DNA into the seminiferous tubules. Control testes transfected with DNA-free liposomes did not show staining at any of the stages examined. To prove expression of exogenous β-galactosidase, expression of β-galactosidase was also confirmed by immunohistochemistry analysis using

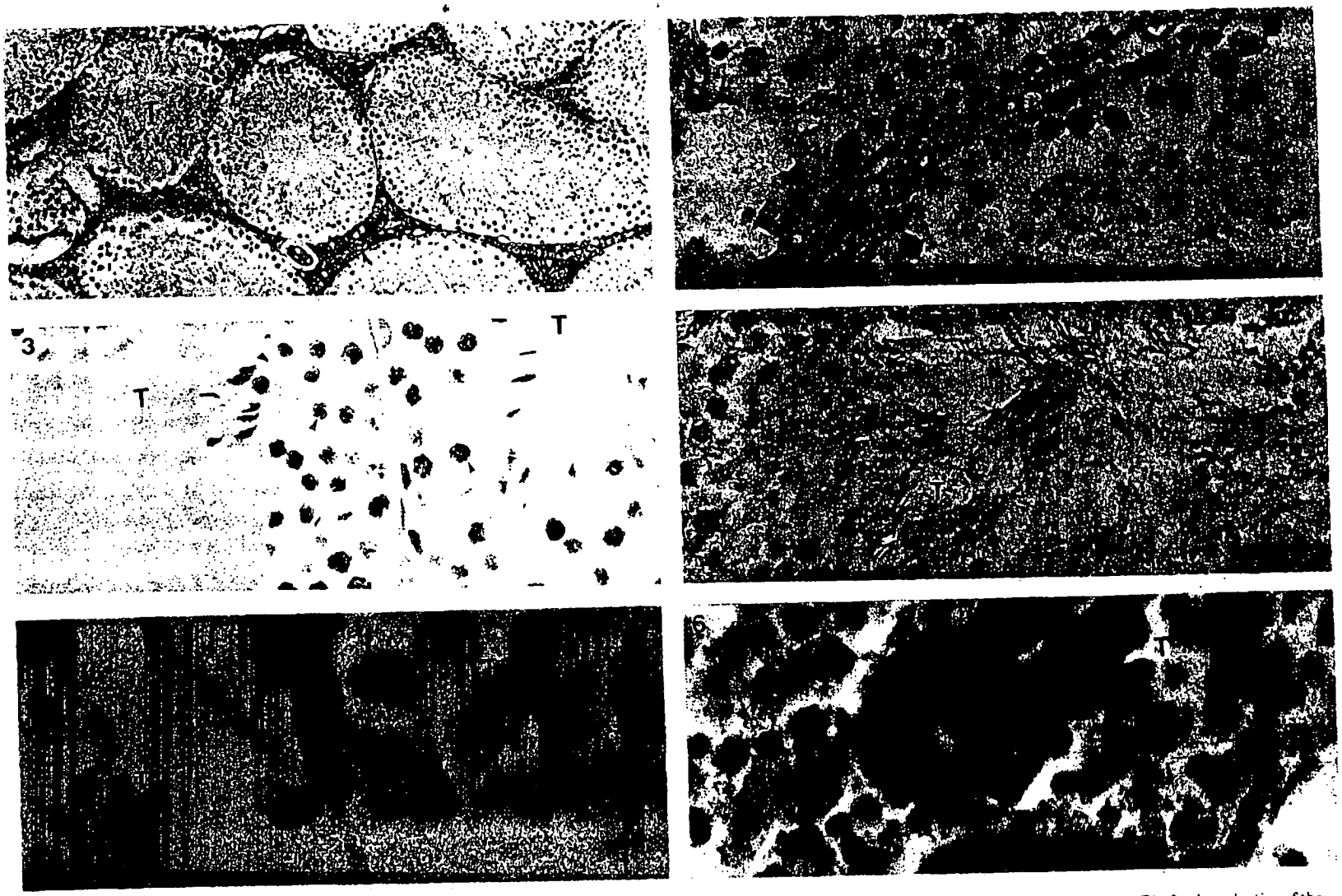

Fig. 5. Immunohistochemistry localization of bacterial LacZ gene in transfected testis. For immunocytochemistry, see Materials and Methods. Testes in different stages of development following busulfan and gene transfer were fixed and labeled by immunohistochemistry with β-galactosidase antibody (1–4) and by X-gal staining (5, 6). After transfection with the bacterial lacZ gene, most of the signal was restricted to Leydig cells (×100, A) and type A spermatogonia (×1,000, B). Six (×1,000, C) and 12 weeks (×1,000, D) after introduction of the lacZ gene, the signal extended to developing spermatocytes and mature spermatozoa. By X-gal staining, some type A spermatogonia partially expressed the lacZ gene (×1,000, E), and some germ cells showed whole staining (×1,000, F). Arrowhead, male germ cells expressing the bacterial LacZ gene.

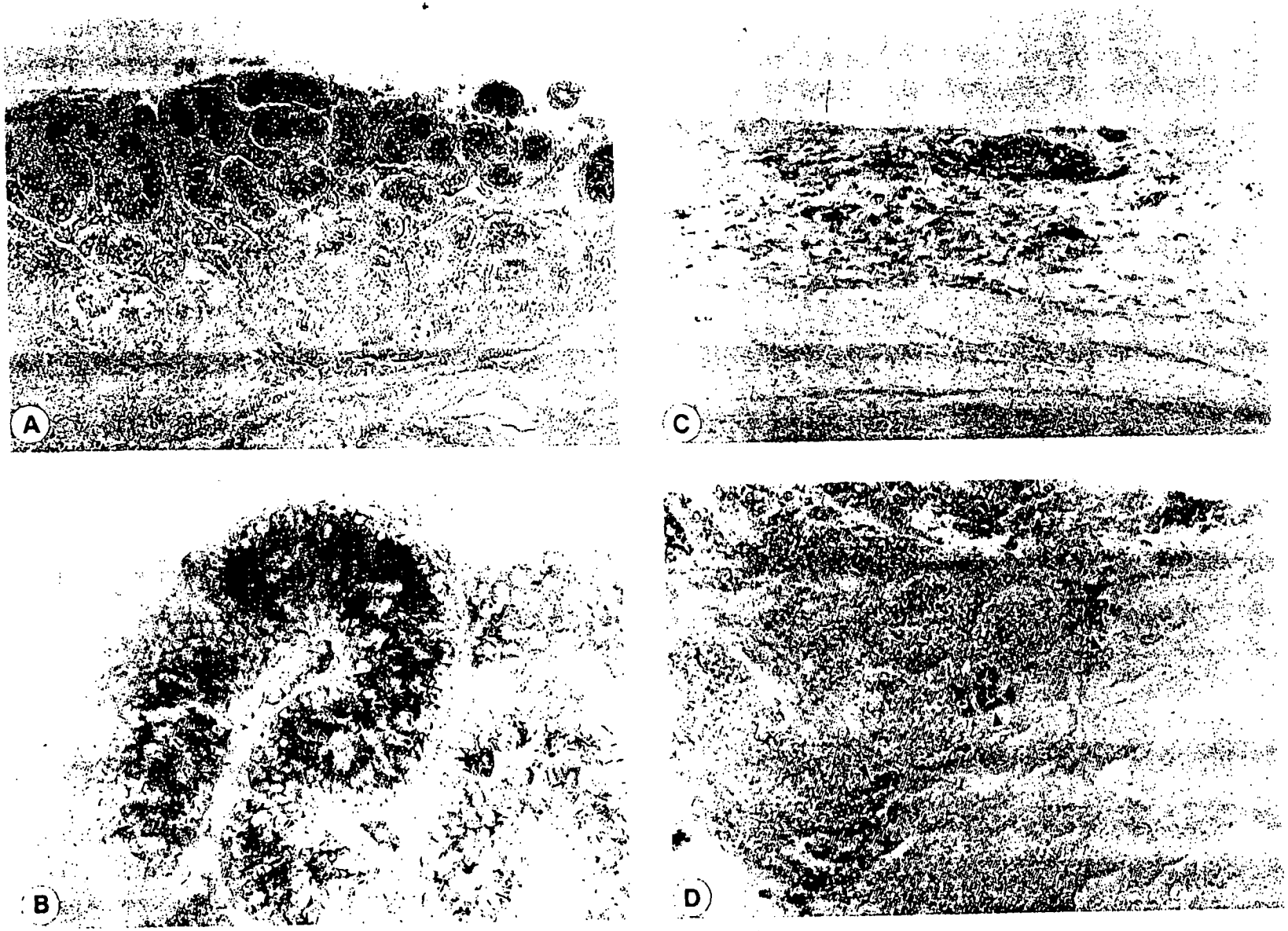

Fig. 6. (Legend on facing page).

matured from the transfected and untransfected male stem cells is relatively low. To overcome this problem, we are going to test the possibility of selecting sperm with exogenous DNA from ejaculated sperm by flow cytometry using an antibody, and we will attempt to improve the efficiency of transgenic livestock production by in vitro fertilization with concentrated sperm harboring foreign DNA. After the completion of this manuscript, we have critical evidence to verify our data (Ogawa et al., 1995), indicating that spermatozoa derived from transfected germ cells can transfer exogenous DNA into oocytes.

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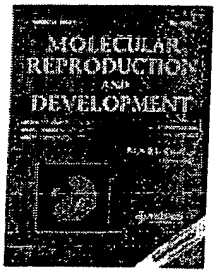

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Transgenics

Liposome-Mediated DNA uptake by sperm cells

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KEYWORDS

DNA transfer • Transgenic animals • Cationic liposomes

ABSTRACT

To investigate the potential use of sperm cells as vectors to transfer exogenous DNA via the fertilization of oocytes into the germ line of mice, we have used liposomes to transfect DNA into the sperm head. Although the DNA transfer into sperm mediated by liposomes was very efficient and no obvious reduction in the fertilization frequency of oocytes could be detected, we were unable to generate transgenic mice by this method.

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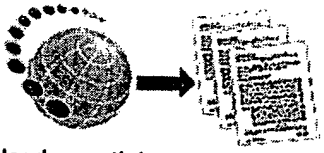

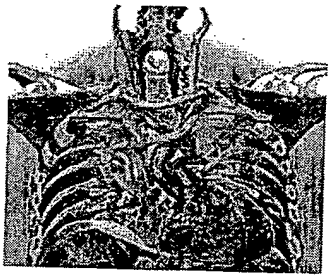

APPENDIX D TO APPELLANTS' BRIEF

RELATED PROCEEDINGS APPENDIX

None

cited documents. Copies of all documents are with the Second Amended Brief as submitted in triplicate.

It is believed that the enclosed Brief should now be in full compliance and the Appeal can proceed.

The Commissioner is authorized to charge any additional fees or refund any overpayment under 37 CFR 1.16 and 1.17 which may be required by this paper to Deposit Account No. 08-1265.

Respectfully submitted,

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CERTIFICATE OF MAILING

I hereby certify that the foregoing Letter in response to the Notification of Non-Compliant Appeal Brief Under 37 CFR 41.37 dated November 27, 2006, a Second Amended Appeal Brief for the Appellants in triplicate, Appendix A, B, C and D in triplicate and copies of cited references in application Serial No. 10/397,438, filed on November 5, 2003, of So Iwata et al, entitled "CRYSTALLOGRAPHY METHODS", are being deposited with the U.S. Postal Service as First Class mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, postage prepaid, on November 30, 2006.

Barbara L Davis

Barbara L. Davis
on behalf of C. G. Mersereau
Attorney for Applicant

Date of Signature: November 30, 2006